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miR-155 Upregulation in Dendritic Cells Is Sufficient To Break Tolerance In Vivo by Negatively Regulating SHIP1

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TLR-induced maturation of dendritic cells (DCs) leads to the production of proinflammatory cytokines as well as the upregulation of various molecules involved in T cell activation. These are believed to be the critical events that account for the induction of the adaptive immune response. In this study, we have examined the role of miR-155 in DC function and the induction of immunity. Using a model in which the transfer of self-Ag–pulsed, TLR-matured DCs can induce a functional CD8 T cell response and autoimmunity, we find that DCs lacking miR-155 have an impaired ability to break immune tolerance. Importantly, transfer of self- Ag-pulsed DCs overexpressing miR-155 was sufficient to break tolerance in the absence of TLR stimuli. Although these unstimulated DCs induced T cell function in vivo, there was no evidence for the upregulation of costimulatory ligands or cytokine secretion. Further analysis showed that miR-155 influenced the level of the phosphatase SHIP1 in DCs and that the lack of SHIP1 in DCs was sufficient to break T cell tolerance in vivo, again in the absence of TLR-induced DC maturation. Our study demonstrates that the overexpression of miR-155 in DCs is a critical event that is alone sufficient to break self-tolerance and promote a CD8-mediated autoimmune response in vivo. This process is independent of the induction of conventional DC maturation markers, indicating that miR-155 regulation of SHIP represents a unique axis that regulates DC function in vivo. ***The Journal of Immunology*****, 2015, 195: 4632–4640.**

Dendritic cells (DCs) play a key role in shaping the T cell repertoire and determining the nature of the T cell response. Studies have clearly shown that immature DCs have the ability to induce T cell tolerance and that mature DCs are critical for activating the adaptive immune response. However, it is clear that DC function can be modulated in several ways, which then have an impact on how DCs influence the immune response. This imprinting in turn has measurable consequences on the immune response (1–5). For example, cytokines or other stimuli have been shown to alter DC function (6–10). These conditioned DCs have the ability to induce regulatory T cells or have an impact on the type of immune response that occurs.

Many studies have shown that pathogen-associated molecules are able to induce DC maturation using different pattern recognition receptors such as the TLRs, C-type lectins, nucleotide-binding domain and leucine-rich repeat–containing receptors, and retinoic acid–inducible gene I–like receptors (11). TLRs detect molecular motifs from a variety of sources including bacteria, viruses, fungi, parasites, as well as synthetic compounds, whereas C-type lectins detect β-glucans and mannans derived from fungal cell walls. Nucleotide-binding domain and leucine-rich repeat–containing receptors generally are stimulated by bacterial pathogens, whereas retinoic acid–inducible gene I–like receptors generally detect viral pathogens. Typical events associated with DC maturation include the induction of proinflammatory cytokines (IL-1, IL-6, IL-12, and TNF-α), IFNs, and the upregulation of costimulatory molecules, all of which contribute to the activation of the adaptive immune response. Studies have shown that miRNAs are also upregulated upon TLR stimulation primarily in macrophages, but the functional consequence of modulating miRNA levels remains to be identified (12–14).

miRNAs are small noncoding RNAs that function by binding mRNAs and blocking their translation (15). Production of miRNAs is dependent on the enzyme DICER in the cytoplasm (16). miRNAs have been shown to have functions in the immune system. In T cells, miRNAs function in Th1/Th2/Th17 skewing (17–20) and regulatory T cell development (21, 22). The development of the B cell and NKT cell lineages also requires the presence of miRNAs (23–25). Recently, the deletion of DICER in the DC lineage using a DC-specific Cre recombinase has shown that DCs develop

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The microarray data presented in this article have been submitted to Gene Expression Omnibus under accession number GSE72716.

The views expressed in this paper do not necessarily reflect those of the Ontario Ministry of Health and Long Term Care.

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Abbreviations used in this article: BMDC, bone marrow–derived DC; cRPMI, complete RPMI; DC, dendritic cell; DC-SIGN, DC-specific ICAM-3–grabbing nonintegrin; FSL-1, synthetic lipoprotein-1; LCMV-gp, lymphocytic choriomeningitis virus glycoprotein; Poly(I:C), polyinosinic-polycytidylic acid; RIP-gp, rat insulin promoter glycoprotein; WT, wild-type.

normally but that Langerhans cells disappear in an age-dependent manner (26).

Although deleting DICER has been a useful tool in determining the function of certain miRNAs, the contribution of individual miRNAs to cellular function remains a key question. Mice lacking miR-155 due to genetic deletion have a variety of immune defects. The T cells in miR-155$^{-/-}$ mice have impaired regulation of Th2 cytokines (17, 19). Regulatory T cells lacking miR-155 show impaired homeostatic expansion (21). The B cells from mice lacking miR-155 appear to develop normally, but have reduced ability to respond to model Ags (17, 19). A recent study has indicated that miR-155 plays a role in autoimmunity by demonstrating that experimental autoimmune encephalopathy cannot be induced in miR-155 knockout mice (27). In DCs, miR-155 has been shown to regulate levels of DC-specific ICAM-3–grabbing nonintegrin (DC-SIGN) (28) and IL-1β (29). DCs lacking miR-155 have been shown to be defective at inducing T cell proliferation (17). Studies on the function of miRNAs have not examined the importance of miR-155 in DCs function in vivo.

We have developed a new protocol to induce CD8$^+$ T cell function by DC transfer into mice with an endogenous T cell repertoire. Our laboratory has previously shown that mice expressing the lymphocytic choriomeningitis virus glycoprotein (LCMV-gp) in β-islet cells of the pancreas under control of the rat insulin promoter glycoprotein (RIP-gp) maintain LCMV-gp–specific T cells in the repertoire. Infection with LCMV has been shown to activate gp-specific T cells, initiating the CD8-mediated destruction of gp$^+$ islet cells and diabetes (30). Recent studies in our laboratory have shown that TLR-matured bone marrow–derived DCs (BMDCs) pulsed with LCMV-gp peptides are able to induce diabetes when DCs are infused into RIP-gp mice. Importantly, when LCMV-gp peptide-pulsed DCs are not matured with TLR signals, the mice do not develop diabetes, confirming the importance of DC maturation and validating this model (31). Therefore, this new model allows us to evaluate the properties of the DCs that are capable of activating CD8$^+$ self-reactive T cells from a normal repertoire in vivo. We have used this model to identify miRNAs that are upregulated in mature DCs and further evaluated the role of miR-155 and downstream pathways that govern the ability of DCs to induce the adaptive immune response in vivo.

Materials and Methods

Mouse strains

Wild-type (WT) C57BL/6NTac mice were obtained from Taconic Farms. miR-155$^{-/-}$ mice were from The Jackson Laboratory (007745). Mice with conditional overexpression of miR-155 (miR-155Tg) were described previously and are a generous gift from Klaus Rajewsky (19). These mice contain a Rosa26 knockin of the miR-155/bic gene preceded by a stop codon flanked by loxP sites and were crossed to mice expressing Cre recombinase under control of the CD11c promoter (Jackson 007567), allowing Cre-mediated excision of the floxed STOP codon and miR-155 expression. Mice were maintained and mouse experiments were performed at the Ontario Cancer Institute animal facility according to institutional guidelines and with approval of the Ontario Cancer Institute Animal Ethics Committee.

DC culture

Bone marrow was flushed from tibias and femurs of mice with HBSS. Bone marrow cells were cultured at 2 × 10^6 cells/ml in 10 ml non–tissue culture–treated dishes in RPMI 1640 containing 10% LPS-free FBS, penicillin/streptomycin glutamine 2-ME (complete RPMI 1640 [cRPMI]), with 40 ng/ml murine GM-CSF (PeproTech). On day 3 of cultures, 10 ml fresh media was added, also containing 40 ng/ml GM-CSF. On days 6 and 8, 10 ml media was removed and centrifuged to collect cells, which were resuspended in 10 ml fresh media containing GM-CSF and added back to dishes. Nonadherent cells were isolated on day 9. DCs were plated in 24-well plates at 2 × 10^6 cells/ml in 1 ml cRPMI with or without the class B CpG ODN1826 (ACGT DNA Technologies, Toronto, ON, Canada) at 10 μM final concentration overnight. The following day, DCs were pulsed with the MHC class I peptides LCMV GP-33–41 (KAVYNFATM) and GP-276–286 (SGVENPGGYCL), both at 10^{-6} M, and the MHC class II epitope GP-61–80 (GLNGPDIYKGVYQFKSVEFD) at 1 μg/ml by adding peptides in 1 ml cRPMI to DC cultures. After peptide pulsing for 2 h, DCs were harvested, and supernatants were collected for cytokine quantification by ELISA. DCs were washed four times in cRPMI, and 2 × 10^6 DCs were infused i.v. into RIP-gp recipients. Blood glucose was measured by Accu-Chek blood glucose meters (Roche).

Array analysis of miRNA expression

BMDCs from WT mice were either stimulated with CpG or left in media alone overnight. Whole RNA was isolated using a mirVana miRNA isolation kit following the manufacturer's instructions (Thermo Scientific). RNA samples were labeled and hybridized to Agilent mouse miRNA 8 × 15K arrays (Agilent Technologies). Data were generated from eight individual samples (four unstimulated and four CpG treated). A heat map was generated using MeV software by the Institute for Genomic Research. Microarray data are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE72716.

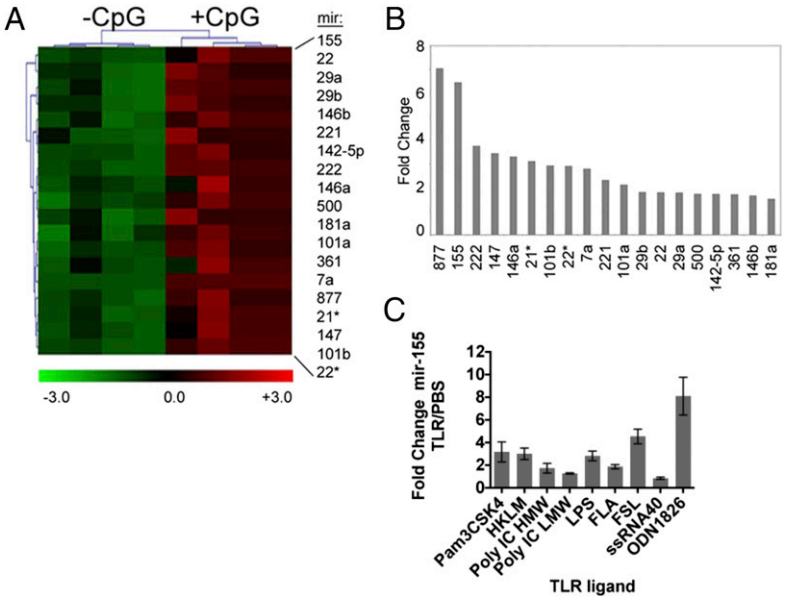

FIGURE 1. Analysis of miRNA upregulation after CpG-induced DC maturation. Expression levels of miRNA were quantified from unstimulated or CpG-stimulated DCs. (**A**) Hierarchical clustering of miRNA signatures revealed a cluster of 19 miRNAs that were increased after CpG stimulation. (**B**) The 19 miRNAs that increased between unstimulated and CpG-treated are listed in descending order of fold change from highest to lowest. Each data point represents normalized data from four individual biological replicates. (**C**) Expression of miR-155 in DC stimulated with the indicated TLR ligands. Graphs display the fold increase in miR-155 between TLR-treated and untreated BMDCs. The experiment was repeated five times, and data shown are mean values of five experiments (± SEM). FLA, flagellin from *S. typhimurium*; HKLM, heat-killed L. monocytogenes; HMW, high m.w.; LMW, low m.w.

Flow cytometry

Individual cell suspensions were stained with Abs for 30 min in PBS containing 2% FBS on ice followed by two washes. The following clones were used: CD11c clone N418, CD80 clone 16-10A1, and CD86 clone GL1 all from eBioscience. Anti-CD40 was clone 3/23 (BD Pharmingen). FcR blocking was accomplished by preincubating DCs with anti-CD16/32 (clone 93; eBioscience) for 30 min before staining with other Abs. Tetramer staining was performed on WBCs isolated by spinning whole blood through Histopaque-1077 (Sigma-Aldrich). Cells were stained with H2-Kb LCMV GP-34–41 tetramer (MHC Tetramer Production Laboratory, Baylor College of Medicine) for 30 min on ice followed by 30 min staining with anti-CD8 (clone 53-6.7; BD Pharmingen). All FACS data were acquired using an FACSCalibur Flow Cytometer (BD Biosciences), and data were analyzed on FlowJo software (Tree Star).

RT-PCR

Total RNA was extracted from 5×10^6 BMDCs using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. cDNA was prepared using random primers and superscript reverse transcriptase (Life Technologies). Primers for bic (19) or TLR9 (32) were used with varying amounts of input cDNA to semiquantitatively amplify the target transcripts. Expression level was quantitated by densitometry using ImageJ software (National Institutes of Health), normalized to β-actin expression.

TLR stimulation and real-time PCR

A total of 2×10^6 BMDCs was incubated with the following TLR ligands at the listed concentration overnight: Pam3CSK4 300 ng/ml, heat-killed *Listeria monocytogenes* 1×10^8 bacteria/ml, polyinosinic-polycytidylic acid [Poly(I:C)] high m.w. 10 μg/ml, Poly(I:C) low m.w. 10 μg/ml, LPS 10 ng/ml, flagellin from *Salmonella typhimurium* 1 μg/ml, synthetic lipoprotein-1 (FSL-1) 1 μg/ml, ssRNA 5 μg/ml, or CpG (ODN1826), all from the Mouse TLR1-9 Agonist Kit (catalog number Tlrl-kit1mw; Sigma-Aldrich). RNA isolation, reverse transcription, and amplification were performed using the TaqMan MicroRNA Cells to C_t Kit (catalog number 4391848; Life Technologies). All signals were normalized to an internal standard, sno-202 RNA. Amplification primers for mmu-miR-155 and sno-202 were from the TaqMan MicroRNA assays (Life Technologies). Amplification and analysis was performed using a ViiA7 real-time PCR instrument (Applied Biosystems).

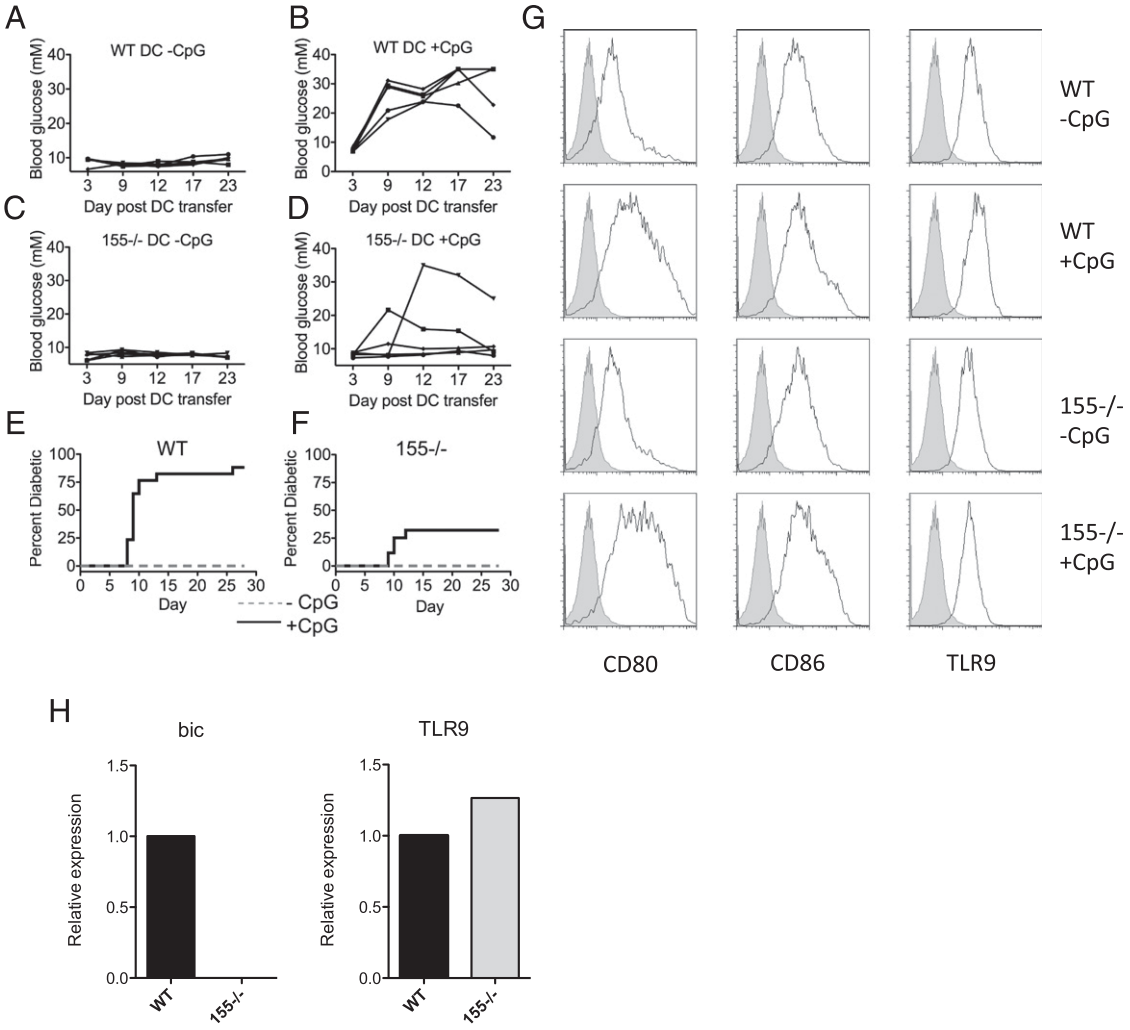

FIGURE 2. Expression of miR-155 in DCs is critical for promoting autoimmunity. RIP-gp mice expressing LCMV-gp on pancreatic islet cells were infused with WT or miR-155$^{-/-}$ BMDCs loaded with LCMV peptides. Blood glucose concentrations were measured at indicated time points after i.v. injection of unstimulated WT (**A**) or miR-155$^{-/-}$ DCs (**C**) or CpG matured DCs from WT (**B**) or miR-155$^{-/-}$ (**D**) mice. Each line shows the glucose levels of an individual mouse. Data represent one experiment of four performed. Incidence of diabetes after infusion with WT DCs (**E**) or miR-155$^{-/-}$ DCs (**F**). DCs were either left untreated (dashed lines) or CpG-treated (solid lines). The *x*-axis displays the time point after DC transfers at which recipients developed diabetes (blood glucose >15 mmol). This graph includes data from four experiments. Unstimulated WT and miR-155$^{-/-}$, $n = 15$; CpG-stimulated WT and miR-155$^{-/-}$, $n = 17$. Differences in diabetes induction between groups of mice receiving WT CpG-treated versus miR-155$^{-/-}$ CpG-treated was significantly different as tested by log-rank (Mantel-Cox) test. $p = 0.0003$. (**G**) Surface expression of CD80, CD86, and intracellular expression of TLR9 in WT versus miR-155$^{-/-}$ BMDCs with or without CpG stimulation were assessed by flow cytometry. The solid gray trace represents isotype control staining; outline trace represents the indicated Ab. (**H**) bic and TLR9 expression level determined by RT-PCR using total RNA isolated from WT versus miR-155$^{-/-}$ BMDC.

ELISAs

Supernatants from overnight cultures of DCs with or without CpG were analyzed for the following cytokines: TNF-α (catalog number 558534; BD OptiEIA), IL-6 (catalog number 555240; BD OptiEIA), IL-12p40 (Ready Set Go catalog number 88-7120-88; eBioscience), and IL-23 (Ready Set Go, catalog number 88-7234-88; eBioscience).

Western blots

DCs were treated with 10 mmol CpG for the indicated times and lysed at 1×10^6 in 50 μl cell lysis buffer (catalog number 9803; Cell Signaling Technologies) containing protease inhibitors (Complete Mini Roche) on ice for 10 min. Centrifuging at 20,000 relative centrifugal force for 15 min at 4°C pelleted cellular debris. Protein was quantitated by the Bradford method (Bio-Rad protein assay dye). A total of 30 μg total protein was mixed with NuPage LDS sample buffer (Invitrogen) and boiled for 10 min. Samples were loaded onto precast 1–12% Bis-Tris gels (NuPage; Invitrogen) followed by transfer to polyvinylidene difluoride membranes and blocking. SHIP1 was detected using rabbit anti-SHIP1 Ab (catalog number D1163; Cell Signaling Technologies). Membranes were stripped and reblotted with anti-actin (catalog number 029K4838; Sigma-Aldrich). Primary Abs were detected using anti-rabbit HRP (GE Healthcare) followed by exposure to ECL plus chemiluminescent substrate (GE Healthcare).

Results

miR-155 is upregulated in mature BMDCs

To identify key miRNAs that are upregulated upon DC maturation, we performed array analysis on BMDCs that were either unstimulated or stimulated with the TLR9 agonist CpG. Importantly, we confirmed that the same pool of CpG-matured DCs were able to induce diabetes in the RIP-gp model after DC transfer in vivo, whereas the CpG unstimulated control DCs were unable to induce diabetes. Therefore, we were certain that these DCs had the ability to differentially induce $CD8^+$ T cells in vivo. Analysis of the miRNA arrays showed that 19 miRNAs increased after CpG stimulation (Fig. 1A), and, in particular, miR-155 showed an ~6-fold increase (Fig. 1B). High miR-155 levels have been observed after LPS stimulation in human monocyte-derived DCs (29), macrophage cell lines (14), as well as in murine macrophages and DCs stimulated with Poly(I:C) or LPS (27, 33, 34). We performed a side-by-side comparison of the effects of a panel of TLR ligands on miR-155 expression (Fig. 1C). miR-155 increased in BMDC after treatment with ligands of TLR4 (LPS), TLR3 [high m.w. Poly(I:C)], TLR2/1 (Pam3CSK4, heat-killed *L. monocytogenes*), TLR2/6 (FSL), TLR5 (flagellin from *S. typhimurium*), and TLR9 (CpG ODN1826). The TLR8 ligand ssRNA40 did not increase miR-155 expression. Our studies demonstrate that miR-155 is increased in DCs to the greatest extent after TLR9 signaling.

Expression of miR-155 in DCs is critical for promoting autoimmunity

To test whether miR-155 was critical for DC maturation, we infused RIP-gp mice with either WT or miR-$155^{-/-}$ DCs, treated with or without CpGs and pulsed with LCMV-gp peptides. When groups of RIP-gp mice received either WT or miR-$155^{-/-}$ DCs that were pulsed with LCMV-gp peptides, but not matured with CpG, there was no increase in blood glucose concentration (Fig. 2A, 2C). In contrast, when RIP-gp mice received WT peptide-pulsed DCs that had been matured with CpGs, the majority of RIP-gp mice developed diabetes (Fig. 2B, 2E). In groups of mice receiving gp peptide-pulsed, CpG-matured miR-$155^{-/-}$ DCs, the majority of mice did not develop diabetes, as indicated by blood glucose remaining <15 mmol (Fig. 2D, 2F). Data from four experiments showed that peptide-pulsed, CpG-stimulated DCs from WT mice induced diabetes in 88% of RIP-gp mice (Fig. 2E), whereas mirR-$155^{-/-}$ CpG-matured DCs pulsed with gp peptides resulted in only 32% of the mice developing diabetes (Fig. 2F). CpG treatment of both WT and miR-$155^{-/-}$ BMDC increased surface expression of DC maturation markers CD80 and CD86 (Fig. 2G). TLR9 expression was unaltered in miR-$155^{-/-}$ BMDCs, as assessed by intracellular Ab staining (Fig. 2G) and RT-PCR (Fig. 2H). Therefore, these data indicate that miR-155 is important for regulating DC function that has an impact in promoting autoimmunity in this model.

Overexpression of miR-155 in DCs without TLR maturation is sufficient to promote autoimmunity

Because miR-155 upregulation was shown to occur after TLR maturation, it was possible that the constitutive upregulation of miR-155 could circumvent the requirement for TLR-induced maturation signals. To test this hypothesis, we generated BMDCs that constitutively expressed high levels of miR-155. Transgenic mice bearing a conditional miR-155 transgene were crossed with mice expressing CD11c Cre (miR-155Tg) (19), and similar experiments

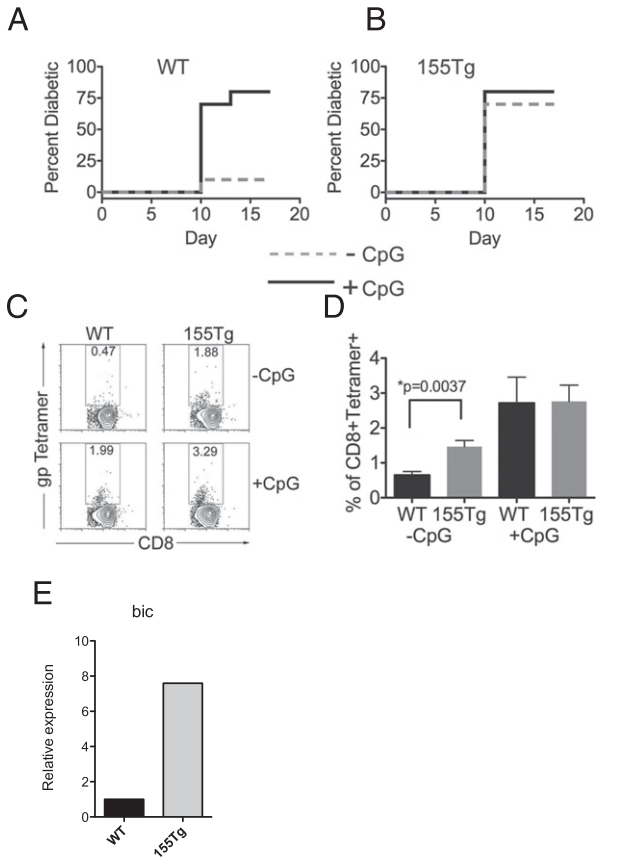

FIGURE 3. DCs constitutively expressing miR-155 are able to trigger a $CD8^+$ T cells response in the absence of TLR stimulation. RIP-gp mice were given 2×10^6 peptide- pulsed DCs from WT (**A**) or miR-155Tg (**B**) mice that had been treated with or without CpG, and blood glucose levels were monitored regularly. (Data are from two experiments; $n = 10$/group.) Differences in diabetes induction in groups of RIP-gp mice that received WT unstimulated DCs versus unstimulated miR-155Tg DCs were significant as tested by log-rank (Mantel-Cox) test. $p = 0.0076$. (**C**) Ag-specific T cell expansion was quantitated in C57BL/6 mice after treatment with peptide-pulsed DCs from WT or miR-155Tg mice that have been treated with or without CpG. (**D**) Average percentages of $CD8^+$ LCMV-GP34 $tetramer^+$ cells. Increased Ag-specific T cells in mice receiving unstimulated miR-155Tg compared with mice receiving WT DCs is statistically significant as determined by Student *t* test. (**E**) bic expression determined by RT-PCR using total RNA prepared from WT versus miR-155Tg BMDC.

were performed with WT and miR-155 Tg DCs. Both CpG-matured WT and miR-155Tg BMDCs that were pulsed with gp peptides induced diabetes in RIP-gp mice (Fig. 3A, 3B). Interestingly, unstimulated peptide-pulsed miR-155Tg DCs induced diabetes in 70% of the RIP-gp recipients, indicating that high levels of miR-155 in DC were sufficient to induce adaptive immunity in the absence of TLR stimulation (Fig. 3B).

We examined whether the miR-155 Tg DCs were able to activate $CD8^+$ T cells by following gp peptide–specific $CD8^+$ T cell expansion. Unstimulated DCs overexpressing miR-155 induced a significantly higher level of Ag-specific T cell expansion compared with unstimulated WT DCs (Fig. 3C, 3D). The extent of miR-155 overexpression was ~7-fold (Fig. 3E), similar to that induced in WT DC by CpG treatment. Collectively, these data demonstrate that the upregulation of miR-155 in DCs is sufficient to alter DC function and induce Ag-specific $CD8^+$ T cell expansion and diabetes in vivo.

Overexpression of miR-155 leads to functional DCs independent of conventional maturation markers

In order to determine whether the overexpression of miR-155 was linked to conventional DC maturation, the phenotype of miR-155Tg DCs was examined by flow cytometry. Interestingly, the levels of CD80, CD86, and CD40 were not spontaneously upregulated on unstimulated DCs overexpressing miR-155 compared with control WT DCs (Fig. 4A), but the maturation markers increased after CpG treatment, similar to WT DCs.

Next, we measured levels of inflammatory cytokines produced by miR-155Tg DCs. Unstimulated DCs overexpressing miR-155 did not produce TNF-α, IL-12p40, or IL-6 at levels comparable with CpG-stimulated DCs (Fig. 4B–D). No IL-10 or TGF-β was detected from WT or miR-155Tg DCs with or without CpG stimulation. Therefore, the overexpression of miR-155 by DCs is able to induce a functional CD8 response without upregulating CD80, CD86, or CD40 or the production of proinflammatory cytokines.

The link between miR-155 and SHIP1 regulates functional DC maturation

Because the upregulation of miR-155 did not coincide with the induction of conventional markers of DC maturation, we decided to examine whether other targets of miR-155 could be linked with the ability of the phenotypically immature DCs to induce a $CD8^+$ T cell response in vivo. SHIP1 (*INPP5D*) has been identified as a direct target of miR-155 by several groups (34–38). SHIP1 is an inositol polyphosphatase that catalyzes conversion of phosphatidylinositol (3,4,5)-trisphosphate to phosphatidylinositol (3,4)-bisphosphate, which has been shown to have functions in immune responses (39). Mice deficient in SHIP1 die months after birth due to infiltration of myeloid cells into the lung (40). We chose to evaluate whether SHIP1 was involved in DC function in this model.

Experiments were done to evaluate whether alterations in the levels of miR-155 had an impact on SHIP1 levels in BMDCs.

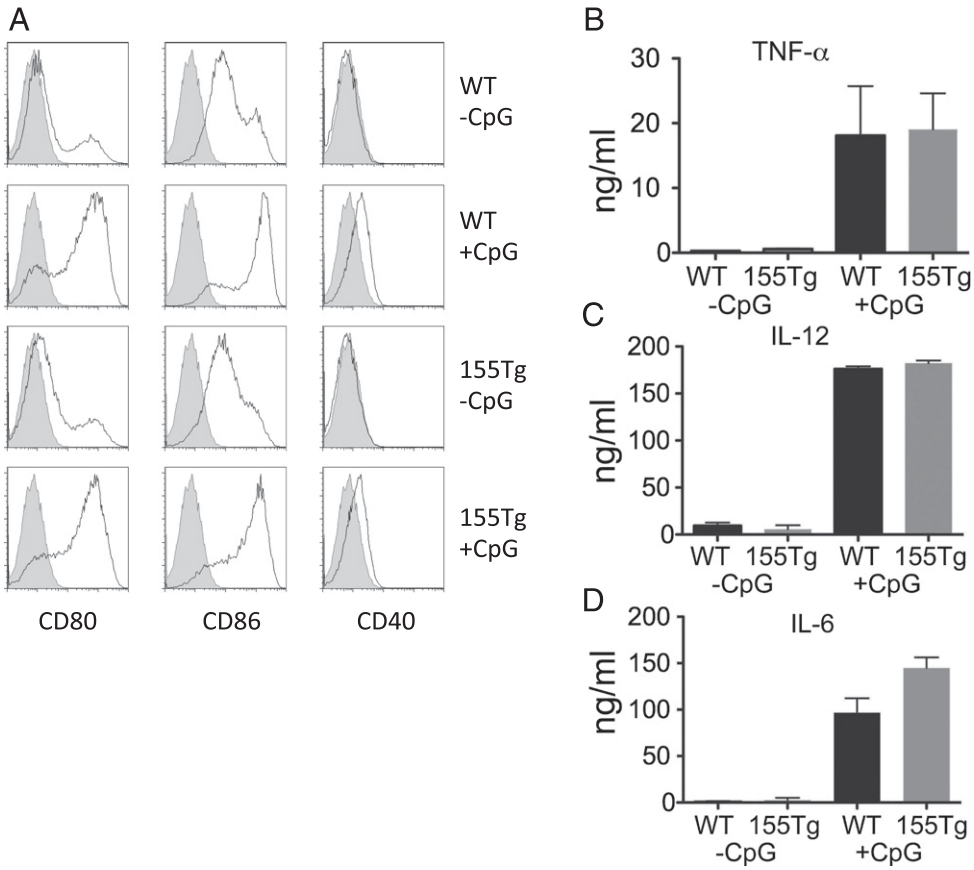

FIGURE 4. Unstimulated DCs that overexpress miR-155 do not upregulate maturation markers or cytokine production. (**A**) The surface expression of various molecules associated with DC maturation was measured on DC from WT or miR-155Tg mice, cultured with or without CpGs. Data are representative of three separate experiments. Gray-shaded areas represent isotype control staining. Black lines represent the intensity of staining for markers on DCs from different genotypes and conditions listed on the right. (**B**–**D**) Proinflammatory cytokines were measured in the supernatant of WT or miR-155Tg DCs with or without CpG treatment. Cytokine levels for TNF-α (B), IL-12p40 (C), and IL-6 (D) were measured. Error bars represent ± SD of duplicate wells. Data represent one representative experiment out of four independent experiments.

Interestingly, Western blot analysis of BMDC lysates showed that levels of SHIP1 protein were ~3-fold higher in DCs lacking miR-155 (Fig. 5A). Conversely, the level of SHIP1 was markedly reduced in miR-155Tg DCs compared with WT DCs (Fig. 5B).

If SHIP1 was an important functional target of miR-155, we would predict that DCs lacking SHIP1 may have the opposite phenotype to DCs lacking miR-155 and a similar phenotype as DCs overexpressing miR-155. To test the ability of SHIP1$^{-/-}$ DCs to induce autoimmunity, we transferred peptide-pulsed SHIP1$^{-/-}$ or WT DC into RIP-gp mice. Fig. 5C and 5D show that both WT and SHIP1$^{-/-}$ DCs induced autoimmune diabetes when matured with CpG and pulsed with peptides. Importantly, SHIP1$^{-/-}$ DCs that were not stimulated with CpG were also able to induce diabetes in RIP-gp recipient mice, whereas unstimulated WT DCs were unable to induce diabetes. These data are consistent with an important role for SHIP1 downstream of miR-155 in regulating the ability of the DC to induce a functional immune response.

In order to determine whether the absence of SHIP1 leads to the upregulation of various maturation markers on DCs, SHIP1$^{-/-}$ DCs were compared with WT DCs. Similar to miR-155Tg DCs, SHIP1-deficient DCs do not have spontaneous upregulation of classical surface molecules associated with DC maturation (Fig. 6A).

We also examined whether the SHIP$^{-/-}$ DCs could induce Ag-specific T cell function in vivo. Unstimulated SHIP1$^{-/-}$ DCs resulted in increased LCMV-gp peptide–specific CD8^{+} T cell expansion and production of the effector cytokine IFN-γ in the spleen of recipient mice (Fig. 6B). Consistent with this result, Ghansah et al. (41) also found that SHIP1$^{-/-}$ APC in lymph nodes also primed better IFN-γ responses by OT-I T cells. These data support an important role for SHIP1 as a critical target downstream of miR-155 in regulating DC function.

We measured the production of proinflammatory cytokines by SHIP1-deficient DCs. Unstimulated SHIP1$^{-/-}$ DC did not produce more TNF-α, IL-12p40, or IL-6 than unstimulated WT DC (Fig. 6C–E). Therefore, like the 155Tg DC, DC lacking SHIP1 are able to induce a functional CD8 response without upregulating CD80, CD86, or CD40 or the production of proinflammatory cytokines.

Discussion

The goal of this study was to identify miRNAs that were critical for regulating DC maturation. In this study, we report that maturation of BMDC by type B CpG resulted in the induction of 19 miRNAs. Interestingly, we did not find that any of the miRNAs represented on the arrays were lower after maturation. We decided to initially investigate miR-155 due to the large fold induction after maturation (Fig. 1B) and the availability of mice that do not express miR-155 or overexpress miR-155 (19).

Although studies have suggested that several miRs are upregulated after TLR stimulation of macrophages and DCs, the importance of miR-155 in DC biology has remained unclear (12–14). Initial studies have shown that the expression of miR-155 in DCs is important for the induction of T cell proliferation using TCR-transgenic T cells in vitro (17), whereas more recent studies did not confirm this finding (27). It has also been shown that miR-155 negatively regulates IL-1 signaling pathways in DCs and could therefore act as part of a negative-feedback loop (29). Another study demonstrated that miR-155 controls levels of DC-SIGN (28). In addition, it has been reported that miR-155 regulates CD115 (CSF-R1) levels and induces apoptosis in DCs (42). It has also been demonstrated in vivo that introduction of miR-155 into DCs via nanoparticles can synergize with CD40 signaling to boost

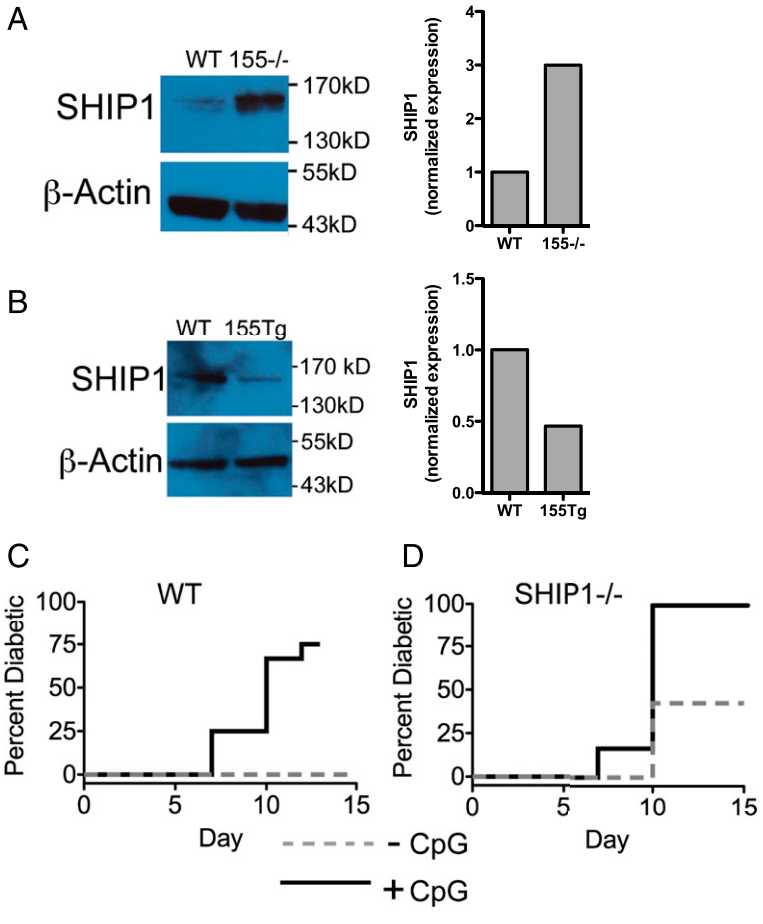

FIGURE 5. SHIP1 influences DC function and the induction of CD8 responses in vivo. Expression of SHIP1 was evaluated by Western blot in DCs from WT and miR-155$^{-/-}$ mice (**A**) and DCs from WT and miR-155Tg mice (**B**). LCMV peptide-pulsed WT (**C**) or SHIP1$^{-/-}$ DCs (**D**) with or without CpG stimulation were transferred into RIP-gp mice, and blood glucose levels were monitored regularly (data represent two separate experiments; n = 10/group). Differences in diabetes induction in groups of RIP-gp mice that received WT unstimulated DCs versus unstimulated SHIP1$^{-/-}$ DCs were significant as tested by log-rank (Mantel-Cox) test. p = 0.0112.

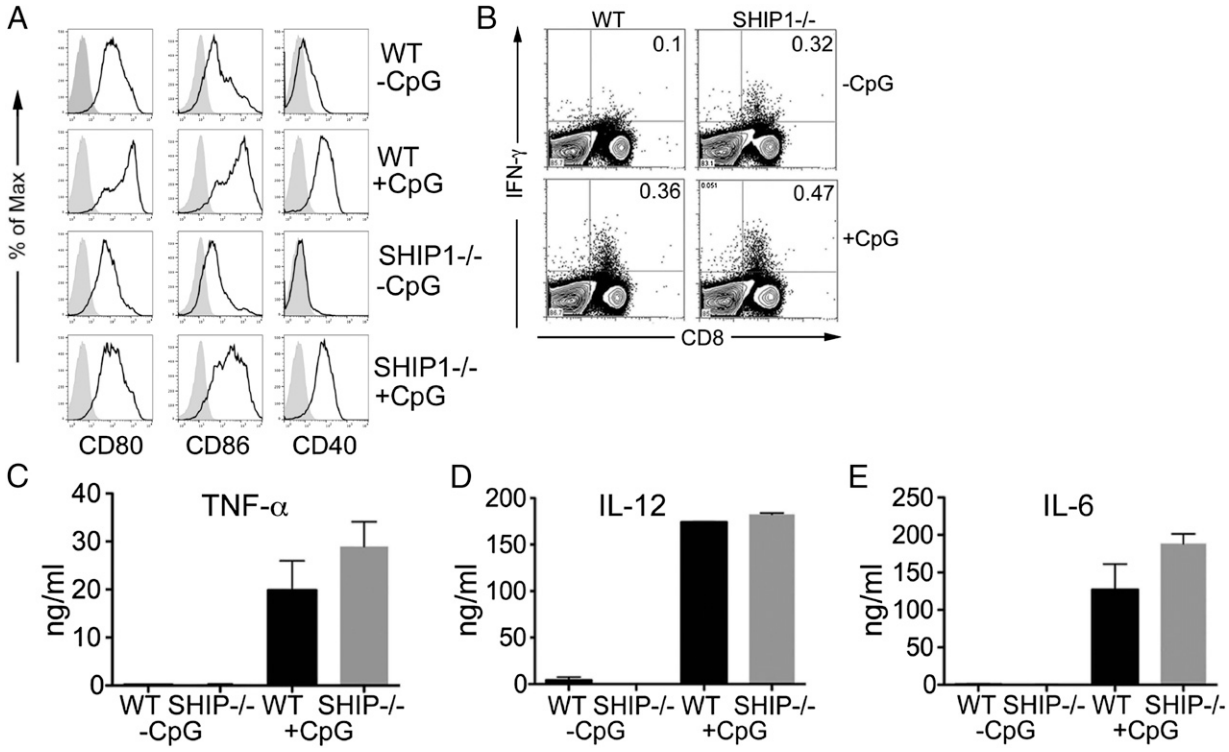

FIGURE 6. Unstimulated SHIP1$^{-/-}$ DCs induce functional T cell responses without upregulating maturation markers or cytokine production. (**A**) The phenotype of unstimulated (−CpG) and CpG stimulated (+CpG) WT versus SHIP1$^{-/-}$ DC was evaluated by flow cytometry by staining with Abs for CD80, CD86, and CD40. (**B**) Splenocytes from mice that received peptide-pulsed CpG-stimulated or unstimulated WT or SHIP1$^{-/-}$ DCs 7 d before were cultured with LCMV-gp peptide and GolgiPlug for 6 h followed by staining for CD8 and intracellular staining for IFN-γ. (**C**–**E**) Proinflammatory cytokines were measured in the supernatant of WT or SHIP1$^{-/-}$ DCs with or without CpG treatment. Cytokine levels for TNF-α (C), IL-12p40 (D), and IL-6 (E) were measured. Error bars represent ± SD of duplicate wells. Data represent one representative experiment out of four independent experiments.

priming of anti-tumor T cell responses in mice (43). These studies all concur on the general premise that miR-155 levels help control the functional phenotype of DC. Although these studies are helpful in determining the phenotypic effects of miR-155 on DC, the natural impact of miR-155 on DCs function in the context of an immune response in vivo remains to be evaluated.

We have demonstrated that DCs lacking miR-155 are impaired in their ability to prime a functional T cell response, leading to a reduction in the incidence of autoimmunity. Also, we find that the upregulation of miR-155 functionally activates DCs in the absence of TLR stimulation and that this alone is sufficient for inducing a CD8-mediated autoimmune response in vivo. Although both CD40 and TLR-matured DCs have been shown to be critical for the induction of autoimmunity (44, 45), the consensus has generally been that this maturation process is linked with the upregulation of critical costimulatory molecules by the DCs as well as the upregulation of proinflammatory cytokines. Our studies have shown that the traditional markers of DC activation (CD80, CD86, or CD40) are not upregulated, nor were the cytokines IL-6, IL-12, or TNF-α. In addition, no changes in BMDC viability or survival were noted (data not shown). Rather, our study has supported a role for SHIP1 in regulating DC function downstream of miR-155.

Over the years, several downstream targets of miR-155 have been identified, including suppressor of cytokine signaling 1 (21, 42), SHIP1 (34–36, 46–49), DC-SIGN (28), CD200 (43), and CD155 (42). Studies investigating the function of SHIP1 in ex vivo–generated and matured DCs have shown that SHIP1 plays a controversial role in DC function. In one study, SHIP1$^{-/-}$ DCs were unable to induce a Th1 response (50), whereas another study showed that purified splenic DCs from SHIP1$^{-/-}$ mice were able to stimulate normal allogeneic T cell responses in vitro (41). Our study uses a stringent in vivo readout to demonstrate that BMDCs derived from SHIP1$^{-/-}$ mice were able to activate T cells and induce a CD8-specific T cell response that results in the induction of immune pathology. Importantly and interestingly, this occurred in the absence of DC maturation signals in SHIP1$^{-/-}$ DCs. Furthermore, the markers that correlate with DC maturation CD80, CD86, and CD40 were not constitutively upregulated on SHIP1$^{-/-}$ DCs, yet these DCs behaved functionally as mature DCs in vivo and could induce diabetes in RIP-gp mice without TLR-induced maturation. Because this phenotype is similar to the phenotype seen in miR-155–overexpressing transgenic DCs, this suggests that miR-155 and SHIP1 regulate the functional status of DCs.

Recent evidence has suggested a link between the deregulation of miRNAs and autoimmunity. Sites of inflammation such as rheumatoid arthritis and atopic dermatitis have been associated with high miR-155 expression (51, 52). Similarly, miR-155$^{-/-}$ mice were shown to be highly resistant to development of experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis (27). Although transfer studies showed a Th17-skewing defect is T cell intrinsic in the miR-155 knockout mouse, additional in vitro data were presented indicating that BMDCs derived from miR-155–deficient mice had minor defects in production of the Th17-skewing cytokines IL-6, TNF-α, and IL-23 after maturation with LPS. If miR-155 was important for induction of IL-6 and TNF-α, the miR-155Tg DCs should constitutively produce these cytokines in the absence of TLR signaling. However, our studies showed that no differences in IL-6 and TNF-α production were found with or without maturation of miR-155Tg DCs. Furthermore, although the data presented by O'Connell et al. (27) indicate that miR-155 does indeed have an impact on DC

function, they did not test the functional ability of miR-155$^{-/-}$ DCs to initiate an immune or autoimmune response. Our study, in contrast, was designed to isolate and study effects on miR-155 on DC functional biology in vivo. Our studies clearly demonstrate that miR-155 expression by DCs has a dramatic impact on the ability to induce CD8 T cell function in vivo.

TLR-induced maturation of DCs has traditionally been thought to be critical for the upregulation of costimulatory molecules as well as the induction of proinflammatory cytokines. Together, these events are believed to be essential for the induction of the adaptive immune response. However, previous studies have shown that autoimmune tissue destruction can occur independently of CD28 costimulation following self-reactive T cell activation by treatments including LCMV, self-peptide plus anti-CD40, and self-peptide plus Hsp70 (44, 53). Also, BMDCs with potent T cell–stimulating activity do not require upregulation of traditional costimulatory molecular markers of maturation (31), and conversely, a CD80^{high}/86^{high} mature surface phenotype of DCs can be associated with immune tolerance induction (6). Unraveling the mechanism(s) that may allow unstimulated, immature DCs to promote autoimmune activation is the subject of ongoing studies. In this report, we further examined the signals that occur downstream of TLR-induced maturation. We present data that miR-155 upregulation alone is sufficient to promote the adaptive immune response in the absence of the upregulation of conventional costimulatory molecules and proinflammatory cytokines. This study, therefore, demonstrates a critical role for miR155 regulating in DC function.

Disclosures

The authors have no financial conflicts of interest.

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